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Opposite role of CD4+CD25+ regulatory T cells and T helper 1 lymphocytes in collateral vessels development

Settore scientifico disciplinare di afferenza

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SUMMARY:

ABSTRACT

Background:

Development of collateral blood vessels is an important compensatory mechanism in response to impaired blood flow due to artery occlusion/stenosis. Several inflammatory cell subpopulations including T lymphocytes are involved in such process. Stabile and colleagues showed that $CD4^+$ and $CD8^+$ T cell subsets are both necessary for efficient arteriogenesis. However the contribution of different $CD4^+$ T cell subsets such as T helper (Th) or the immunosuppressive regulatory $CD4^+CD25^+$ T cells (Treg) in collateral responses is largely unknown.

Aim of the study:

The present study aims at characterizing the role of Th1, Th2 and Treg in the growth of collateral vasculature following artery occlusion.

Methods:

A mouse model of peripheral ischemia by surgical femoral artery occlusion (hindlimb ischemia model) in CD4 knockout mice (CD4-/-) lacking CD4 T cells was used. These mice displaying impaired collateral responses, were used as recipients in rescue experiments in which was studied the effect of i.v. injection (reconstitution) of different $CD4^+$ T cell subsets purified from wild-type animals. Blood perfusion and limb function impairment were measured over

time by laser Doppler imaging and by a semiquantitaive ambulatory impairment clinical score, respectively.

Results:

Reconstitution with $CD4^+$ naïve (composed of effector cells) but not memory T cells (comprising both effector and Treg) restored the capacity to recover blood flow and limb function of CD4-/- mice. In addition $CD4^+$ memory T cells depleted of the Treg cell subset were effective in restoring collateral response in recipient animals. Furthermore *in vitro* polarized Th1 but not Th2 cells supported the recovery of blood perfusion and limb function following femoral artery occlusion.

Conclusions:

Altogether these data indicate that Th1 but not Th2 cells act as positive regulators of collateral vessels development while Treg cells inhibit such response. The identification of specific circulating cellular subsets playing either as positive or negative regulators of collaterals growth may be exploited for the identification of novel easily detectable molecular markers for the assessment of the individual capacity to compensate vascular occlusion and for the design of novel therapeutic approaches for peripheral and cardiac ischemic diseases.

GENERAL INTRODUCTION:

Peripheral artery diseases are the leading causes of death in developed countries. Development of collateral blood vessels is an inherent compensatory mechanism in response to impaired blood flow. Arteriogenesis, consisting in the remodeling of pre-existing small arterioles into larger vessels, is the critical process leading to increased collateral flow. The individual ability to mount collateral circulation following artery occlusion/stenosis markedly influences the clinical consequences of ischemic diseases such as, for istance, intermittent claudication or angina pectoris. Coronary artery disease and peripheral obstructive arteriopathy strictly correlate to senescence, dyslipidemia, diabetes, etc. These factors are often associated with dysregulation of immune function. To date, effective pharmacological approaches to enhance collateral circulation are lacking, suggesting that it is very important to better understand the cellular and molecular events regulating arteriogenesis.

The idea of promoting angiogenesis is still an unexplored therapeutic approach for the treatment of ischemic tissues endowed with a terminal blood supply, such as myocardium and limbs.

Ischemic cardiovascular and cerebrovascular diseases represent the leading causes for mortality in Western countries (Lifton et al., 2001). Functional recovery of ischemic tissues/organs relies on the re-establishment of blood perfusion through collateral networks that supply oxygenated blood to specialized cells. In response to ischemic insults, most tissues display notable

capacities to compensate low levels of oxygen by means of vascular remodeling, angiogenesis, vasodilation, arteriogenesis, and hematopoiesis. Fine molecular mechanisms control these important processes of oxygen compensation, and involve induction of a distinct set of gene products regulating the transcriptional activation of vascular and hematopoietic modulators (Boutin et al., 2008; Makino et al., 2001). For instance, hypoxia inducible factors 1 and 2 (HIF-1 and HIF-2) are often upregulated in order to promote the expression of several target genes such as nitric oxide (NO), vascular endothelial growth factor (VEGF) and erythropoietin (EPO), which in turn induce angiogenesis, vasodilation, and hematopoiesis (Boutin et al., 2008; Makino et al., 2001; Cao, 2009; Lendahl et al., 2009).

However, acute responses to hypoxia may not necessarily be beneficial for the functional recovery of ischemic tissues. For example, HIF-induced VEGF potently induces vascular permeability, which promotes the environmental conditions leading to edema in ischemic tissues (Eriksson et al., 2003; Senger et al., 1983). Increased angiogenesis occurring in hypoxic areas and supported by increased VEGF expression reduces the average distance of cells from the nearest blood vessel. As oxygen diffusion is inversely correlated with such distance, angiogenesis is essential for re-establishing normoxic conditions. However the formation of new capillaries also increases peripheral resistance. Hence, perfusion recovery is achieved only if a concomitant increase in the number of conductive vessels takes place.

Differently, arteriogenesis is not driven by hypoxia but rather occurs in response to increased fluid shear stress as a consequence of vessel occlusion,

and consists in the remodeling and growth of collateral arterioles from pre-existing anastomoses. Consequently the blood flow, downstream the site of vessel occlusion, is recovered.

VASCULOGENESIS A GENERAL OVERVIEW:

In vertebrates organogenesis, blood vessels represent one of the first anatomical structures reaching a functional state (Risau & Flamme, 1995); such event is a fundamental prerequisite for the evolution of complex organisms which develop an increasing need for oxygen and nutrients and require the removal of metabolic waste products.

Vascular growth is a complex phenomenon which continues also in adult life, both in physiological and pathological conditions, either in the form of cyclic processes such as decidual arteriogenesis, or in response to acute or chronic stress, occurring in atherosclerotic vascular disease, chronic inflammation, in the hypoxic environment of growing malignancies, or in response to physical training (Hänze et al., 2007).

Importantly, most major causes of disease in humans involve changes in tissue vascularization and oxygen availability (Semenza, 2007).

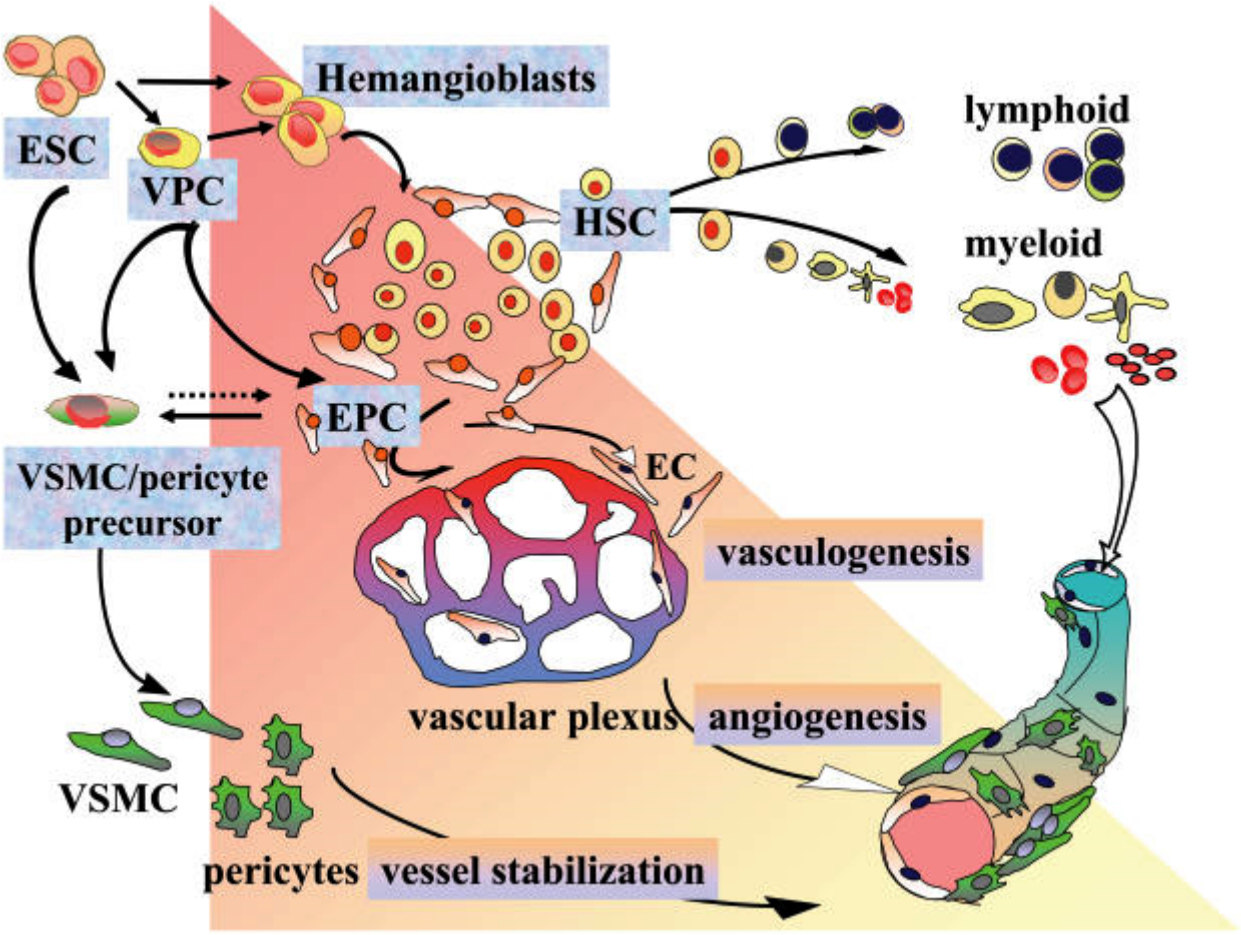
A detailed understanding of the mechanisms regulating vasculogenesis in physiologic and pathologic conditions, represents the basis for the design of therapeutic approaches involving vascular growth stimulation or inhibition.

The formation of the earliest blood vessels (around embryonic day E18 in humans, E6.5 in mice) occurs through vasculogenesis in all mammals.

Vasculogenesis relies on complex serial events including mesoderm formation, the establishment of the endothelial cell (EC) lineage (so-called angioblasts), their organization into cord-like vascular structures, lumen formation, and finally the organization of early vessels into vascular networks. The vascular system and the primordial blood cells originate from the mesoderm, one of the three germ layers that are formed during early embryogenesis. (Risau & Flamme, 1995). The term vasculogenesis indicates the process leading to the formation of the earliest primary vascular network, consisting of precursors of major vessels connected by the honeycomb-like plexus, (Yancopoulos et al., 2000).

It is debated whether vasculogenesis occurs also in postnatal life (Luttun & Carmeliet, 2003; Péault, 2010). In adult circulation, cells expressing molecules such as CD34 and VEGF-2R characteristic of embryonic hemangioblasts, as well as VEGF, granulocyte macrophage colony-stimulating factor (GM-CSF), basic fibroblast growth factor (bFGF), and insulin-like growth factor (IGF-1) mediating their mobilization and/or differentiation (Pelosi et al., 2002; Carmeliet, 2000), have been detected.

On the other hand, other authors restrict vasculogenesis to a well defined time frame occurring during early embryogenesis (Hänze et al., 2007; Semenza, 2007) and consider inappropriate the use of such term to describe adult blood vessel development (Ferguson et al., 2005). In addition although environmental factors may modify the vasculogenic program in the embryo, genetic predetermination governs vasculogenesis.



Vasculogenesis and angiogenesis during development and differentiation. The mesodermal progenitor cells derived from embryonic stem cells (ESC) differentiate into various vascular and hematopoietic cells. Vascular progenitor cells (VPC) are bi-potentials that can differentiate into either pericyte/vascular smooth muscle cells (VSMC) or endothelial cells (EC) lineage. Hemangioblasts generate the blood island composed of endothelial progenitor cells, also called angioblasts (EPC) and hematopoietic stem cells (HSC). EPC differentiate into EC and form vessel tubes, whereas HSC further differentiate into lymphoid/myeloid lineage. Nascent vessels are stabilized by vascular mural cell coverage and extracellular matrix.

Adapted from: **Furuya M. et al., Vasc Health Risk Manag. 2005**

ANGIOGENESIS:

Angiogenesis comprises capillary growth either by sprouting from or by splitting of pre-existing vascular structures (intussusception) (Risau, 1997).

During development, angiogenesis establishes a functional circulation in previously avascular tissues. While vasculogenesis forms the primary vascular plexus in endo- and mesoderm-derived organs, ectoderm-derived tissues such as the brain, receive blood supply by means of angiogenesis (Pardanaud et al., 1989).

Physiological angiogenesis is a tightly controlled process critically dependent on the interaction of the endothelium with the environment and regulated by cytokines and growth factors (Chung et al., 2010).

Sprouting angiogenesis is initiated by vascular leakage and local degradation of the basal membrane (BM) of the mother vessel. In response to local hypoxia, the release of proangiogenic factors such as VEGF induces proteolytic degradation of the extracellular matrix (ECM) by ECs membrane metalloproteinases (MMPs). In this context, ECs proliferate and migrate toward gradients of proangiogenic factors. Vessels subsequently mature and finally acquire tissue-specific properties (Risau, 1997; Carmeliet, 2000; Fischer et al., 2006).

A preceding NO-mediated local increase in vascular diameter has especially been described for pathological angiogenesis (Carmeliet, 2000). Leaking plasma proteins then form a scaffold for migrating ECs in a

platelet/endothelial cell adhesion molecule-1(PECAM-1/CD31)/Vascular endothelial-Cadherin/Src kinase- mediated process (Eliceiri et al., 1999).

First of all, VEGF leads to increased permeability. Subsequently, angiopoietin-2 (Angpt-2), the physiological antagonist of Angpt-1, destabilizes the matrix, with a subsequent partial degradation of the BM and surrounding ECM as well as proteolytic activation of angiogenic growth factors (Carmeliet, 2000). Angpt-2 activated MMPs and proteolytic enzymes liberate matrix-bound pro- and antiangiogenic mediators in an orchestrated sequence, and regulate the biological activities of factors such as VEGF by proteolytic cleavage (Fischer et al., 2006).

Next, some ECs acquire an activated phenotype characterized by upregulated expression of adhesion molecules in response to inflammatory mediators and hemodynamic forces, active mitosis and migration.

Hypoxia drives angiogenesis through intracellular stabilization of the hypoxia-inducible factor (HIF-1) and subsequent induction of VEGF expression.

Importantly, leukocytes play an important role in angiogenesis. In fact, monocytes/macrophages function as cellular regulators of angiogenesis, and are recruited to sites of active angiogenesis by chemokines such as VEGF-A and placental growth factor (PIGF) (Bernardini et al., 2003; Barleon et al., 1996) to exert subpopulation-specific effects (Carmeliet, 2003).

Both vasculogenesis and angiogenesis lead to chord-like EC alignments (Zeeb et al., 2010), which form vascular networks by fusion, giving rise to anastomosis and lumen formation.

Lumen formation occurs either by intracellular vacuoles association or by formation of an internal extracellular lumen from ECs solid cilinders. These phenomena seem to differ in physiological and pathological conditions; sprouting and lumen formation can occur either at the same time or in sequence, and pathways appear to be context-specific. In fact, lumenization based on vacuolation has been for istance observed epecially for isolated ECs lacking cell–cell contacts (Iruela-Arispe & Davis, 2009). Recent data supported the model of extracellular lumen origin. VEGF is necessary for lumen formation (Iruela-Arispe & Davis, 2009). Angpt-1 increases vessel diameter, while Thrombospondin-1 and tubedown-1 reduce it (Fischer et al., 2006). Once functional vessels have been generated, mechanical factors displace genetic pre-specifications in determining lumen diameter (Jones et al., 2006).

Angiogenic vascular morphogenesis enters its resolution phase when the activated, proteolytic phenotype of the invading tip cell is "turned off" by EC-pericyte contacts. Tip cell MMP activity is downregulated and replaced by an activation of its inhibitors TIMP-2 and -3 (Saunders et al., 2006). A downregulation of major angiogenic factors such as VEGF, and a rise in local levels of plateled-derived growth factor (PDGF), transforming growth factor β1 (TGFβ1) and Angpt-1 occur (Chung et al., 2010).

During angiogenesis, endothelial cells are activated by ischemia and grow in the direction of angiogenic signals. The endothelial cells fuse and develop a lumen, thereby forming a new, small capillary vessel.

Adapted from: **van Oostrom M.C. et al., J. Leuk.Biol. 2008**

ARTERIOGENESIS:

While angiogenesis refers to the process of growing new blood vessels, arteriogenesis involves remodeling of existing arterial vessels (Cao, 2009; Tirziu et al., 2005). In fact, arteriogenesis is characterized by the increase in caliber of arteriolar anastomoses to collateral vessels. These vessels can grow considerably, in order to re-establish blood perfusion following artery stenosis or occlusion. While angiogenesis is triggered by hypoxia, arteriogenesis is independent of oxygen levels and usually occurs in a non-hypoxic environment. It is instead promoted by changes in fluid shear stress (FSS) forces sensed by the vascular endothelium (Heil, et al., 2004).

Hence the differences between angiogenesis and arteriogenesis reside in the triggering stimulus (ischemia vs shear stress), the mechanism involved and the result. In fact while capillaries are structurally simple tubes made of endothelial cells, with no additional wall structures, arteriogenesis implies a complex process including coordinated proliferation of endothelial, smooth muscle and fibroblasts accompanied by extensive tissue remodeling.

Collaterals originate, at least in part, from pre-existing vessels located proximal to the site of arterial obstruction and therefore proximal to the ischemic tissue (Heil, et al., 2004; Schaper & Buschmann, 1999). Although in normal conditions little or no flow occurs in these vessels, following sudden occlusion or a slow progressing stenosis of their companion main conductive artery, flow increases along with shear stress and promotes their opening. Hence, the collateral vessel wall now senses various pronounced mechanical forces, and augmented blood flow directly increases fluid shear stress (FSS). It can be estimated using the following equation, due to Newtonian fluid dynamics:

$$\tau = 4\eta Q/\pi R^3$$

This equation includes blood viscosity (η) and the internal radius of the vessel (R), indicates that increased blood flow (Q) will directly result in increased FSS (τ)(Cox ,1979).

Moreover, the wall of the collateral arterioles is influenced by pressure-related forces like longitudinal-, circumferential-, and radial wall stresses. Tension of the vessel wall, structurally weakened by matrix digestion and by intravascular pressure, increases the circumferential wall stress, a known activator of smooth muscle cells (SMCs) proliferation (Scheel el al., 1979). Mature collateral vessels differ only in minor histological aspects from normal arteries of the conductance type: they show a thicker muscular component and contain more collagen. Proliferation of EC and SMC, and ECM remodeling

determine an increased lumen size with subsequent enhanced perfusion to the ischemic tissue (Schaper & Scholz., 2003).

Collateral response can be considered an inflammatory process involving the infiltration of different leukocyte subsets. Compelling evidences indicate that monocyte/macrophages as well as various lymphocyte subsets including cytotoxic and helper T cells as well as natural killer (NK) cells play essential role as positive regulators of such response (van Weel et al., 2007).

Animal models for the study of arteriogenesis have been developed in rodents such as rabbit and mouse. The different nature of the triggering stimulus (hypoxia for angiogenesis and FSS for arteriogenesis) results in different anatomical localization of sites where capillaries or arterioles grow. In the murine model of hindlimb ischemia through surgical ligation of the femoral artery, no overt evidence of ischemia has been found at site where collaterals are actively developing (adductor muscle). This indicates that ischemia plays either no or at most a minor role in gene expression and that collateral vessels developing proximally to an arterial obstruction do not require the local expression of either VEGF or HIF1 (Lee et al., 2004).

Growing collaterals are characterized by the presence of infiltrating leukocytes concentrated in the perivascular zone. When the expression profiling technology was adopted to investigate temporal patterns of gene expression during collateral development in the mouse model of hindlimb ischemia, the apparently prominent role played by genes modulating inflammatory response has been observed. These genes were differentially regulated, with many showing extremely high levels of transcriptional activity.

Endothelial cells are the major sensors of shear stress changes that are transduced into biochemical signals. Integrins (Jalali et al., 2001), tyrosine receptor kinases (Chen et al., 1998; Jin et al., 2003), G protein-coupled receptors (Chachisvilis et al., 2006), and ion channels (Davies et al., 1997; Nilius & Droogmans 2001) have been proposed to act as shear stress sensors on the endothelial cell membrane. These signal transduction cascades lead to the activation of endothelial cells. FSS induces multiple responses at the collateral endothelium. One of the early signs of endothelial activation is cell swelling and the expression of multiple genes involved in the recruitment of circulating leukocytes including chemokines, cytokines and adhesion molecules (Scholz et al., 2002; Hoefer et al., 2002; Unger et al., 1994; Fernandez et al., 2003; Deindl et al., 2003; Rissanen et al., 2003; Emanueli et al., 2002; Liu et al., 2003; Lee et al., 2004). In fact, activated endothelial cells display increased expression of intercellular adhesion molecule (ICAM-1) (Hoefer et al., 2004) and vascular cell adhesion molecule-1 (Scholz et al., 2000). In addition EC undergoing increased FSS release NO (Cai et al., 2004) as well as inflammatory chemokines including (CCL-2) and cytokines (such as tumor necrosis factor-α [TNF-α] and GM-CSF) (Hoefer et al., 2002; Luo et al., 2006), (Kosaki et al., 1998). Thus, the collateral endothelium switches from a quiescent vessel layer with very low adhesion tendency into a highly activated one, which is now supporting attraction, activation, and adhesion of leukocytes.

Infiltrated monocytes/macrophages are present in perivascular zone. Monocytes/macrophages invading areas where collaterals grow, possess at

least three key features relevant to promoting arteriogenesis: 1) responsiveness to chemokines and chemoattractants expressed by endothelial cells in response to increased fluid shear stress force (e.g. CCL-2); 2) ability to promote fibroblasts, endothelial and smooth muscle cells proliferation through the release of relevant amounts of appropriate growth factors such as VEGF, bFGF and PDGF; 3) capacity to participate to extracellular matrix remodeling through the secretion of matrix metalloproteinases.

Among chemokines involved in this process, a dominant role has been attributed to CCL-2. CCL-2 is a member of the CCL family of chemokines and it has been shown to be released by endothelial cells stimulated by interleukin-1 (IL-1) or TNF-α. Upon engagement of its cognate receptor CCR2 expressed on memory T cells, dendritic cells and monocytes, CCL-2 contributes to the recruitment of these cells to sites of tissue injury, infection, and inflammation. Moreover, in different animal models of femoral artery ligation the infusion of CCL-2 in the collateral circulation leads to increased number of monocytes surrounding growing collaterals and improved collateral flow (Ito et al., 1997; Hoefer et al., 2001; van Royen et al., 2003; Voskuil et al., 2003). On the other hand, mice genetically deficient for CCR-2 displayed reduced monocyte infiltration and decreased arteriogenesis following artery occlusion (Heil et al., 2004).

As well as CCL-2, other molecules can serve as chemoattractants favouring monocyte accumulation around growing collaterals vessels: monocytes express the VEGF receptor flt-1 also referred as VEGF receptor-1 (VEGFR-1).

Ligand-induced triggering of membrane VEGFR-1 elicits monocyte upregulation of integrins involved in the interaction with endothelial cells (Heil et al., 2000) and stimulates monocyte chemotaxis *in vitro* (Barleon et al., 1996). In a rabbit model of femoral artery ligation, increased monocyte recruitment and improved collaterals growth was obtained by local infusion of placenta growth factor (PlGF), a selective agonist of the VEGF receptor flt-1. Notably, the proarteriogenic effect of PlGF was abrogated in animals pharmacologically depleted of monocytes (Pipp et al., 2003). Furthermore increased TGF-β1 expression was found in growing collaterals. Local application of exogenous TGF-β increased collaterals growth and collateral conductance. In addition, *in vitro* treatment of monocytes with TGF-β upregulated the expression of the αMβ2 integrin (also known as complement receptor 3 or MAC-1 antigen) involved in cell adhesion to the endothelium, suggesting that TGF-β1 might foster arteriogenesis by improving monocyte recruitment (van Royen et al., 2002). It is generally accepted that infiltrating monocytes and macrophages orchestrate the remodeling process that leads to the formation of collateral arteries and contribute to the peculiar inflammatory environment of the anastomoses (Arras et al., 1998). They do so by 1) producing a pool of factors sustaining inflammation such as TNF-α thus increasing leukocyte recruitment, 2) upregulating matrix metalloproteinases (MMPs) involved in the remodeling of extracellular matrix required for the formation of new structures 3) stimulating the proliferation of endothelial and smooth muscle cells through secreted b-FGF (Baffour et al., 1992; Deindl et al., 2003), PDGF (Arras et al., 1998; Cao et al., 2003) and VEGF.

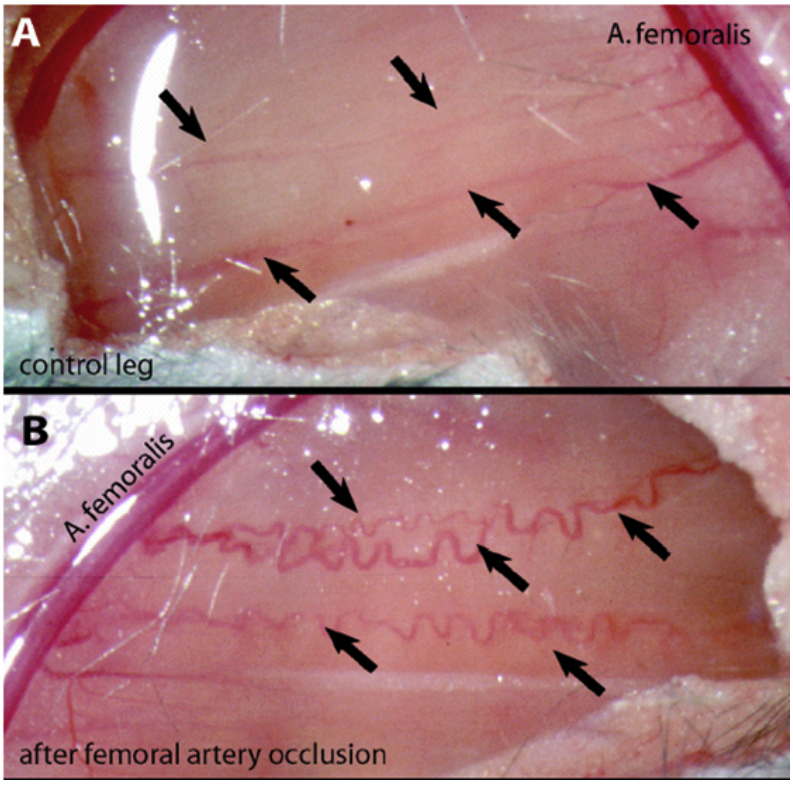
At this point, monocytes have taken over the role of leading characters in the arteriogenic process from the endothelial cells. MMPs secreted by macrophages (Bergmann et al., 2006) break down the surrounding tissue, i.e., the extracellular matrix (ECM) and the internal elastic lamina consequently allowing monocytes to invade the vascular wall. This further enables paracrine signaling between the perivascular cells, such as pericytes and smooth muscle cells (SMC) and the endothelium, hence creating space for the growing vessel (Polverini et al., 1997).

As a result of the loss of extracellular matrix and intravascular pressure, smooth muscle cells slip away from each other. This allows the vessel to enlarge, acquiring a vein-like appearance (Cai et al., 2000). Endothelium specific platelet-derived growth factor B (PDGF-B) and PDGFR expression on SMC play an important role in subsequent migration and recruitment of SMC to the subendothelial space, where they form the neointima layer (Wolf et al., 1998; Hellström et al., 1999). The proliferating smooth muscle cells dispose themselves around the growing vessel and exhibit a "synthetic" phenotype, defined by production of ECM, collagen, and elastin (Buschmann et al., 1999). In this manner, the SMC reconstitute the internal elastic lamina and the tunica media. Growth factors such as bFGF and TNF-α are secreted by monocytes already present in the tissue, and facilitate the proliferation of vascular cells.

The final phase in collateral growth is maturation of the vessels. This is characterized by reduced proliferation, migration and proteolytic activity,

and the differentiation of SMC to the contractile phenotype (Scholz et al., 2000).

During arteriogenesis collaterals can grow up to 20 times in diameter. (Wolf et al., 1998). Since collateral vessels grow in length as well as in width, the expanding vessel arranges itself in loops and turns to accommodate the extra length. This gives the vessels a typical corkscrew pattern (Heil et al., 2006) and causes a remarkable loss of dynamic forces of the fluid circulating inside.



Arteriogenesis and angiogenesis are both necessary after ischemia to compensate the insufficient blood supply and maintain tissue function. In order to face an acute ischemic insult, arteriogenesis is probably a more effective mechanism to rescue tissue and organ function whereas the

formation of new vascular networks may represent a delayed phase to compensate tissue ischemia.

In arteriogenesis, circulating leukocytes (green) are attracted to the activated endothelium. They assist in enlarging collateral anastomoses. Activated endothelial cells (blue), activated vascular smooth muscle cells (yellow), quiescent endothelial cells (gray), quiescent smooth muscle cells (brown).

Adapted from: **van Oostrom M.C. et al., J. Leuk.Biol. 2008**

ROLE OF T LYMPHOCYTES SUBSETS IN THE ARTERIOGENIC RESPONSE:

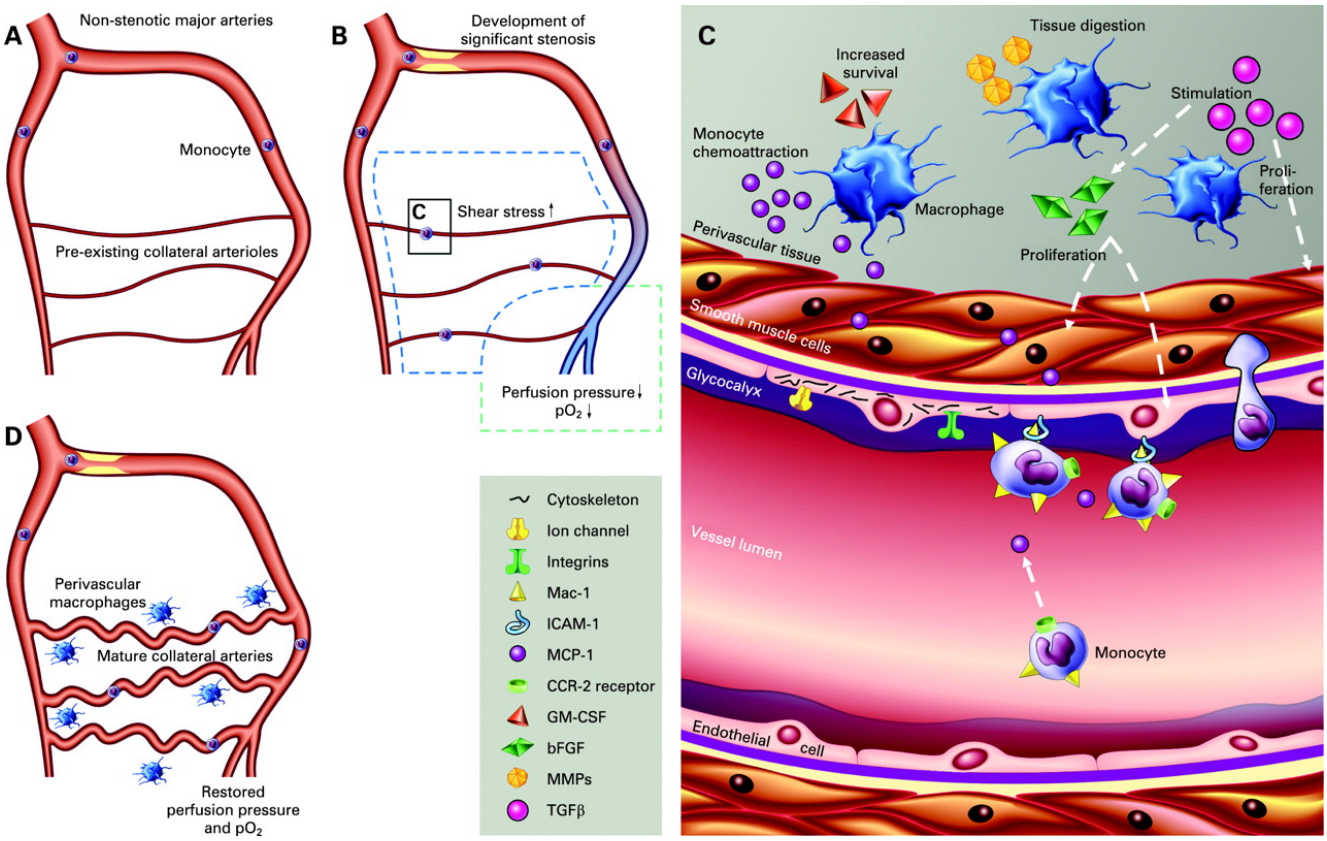
Other circulating cells are also known to participate in the arteriogenic response. Lymphocytes are observed frequently in the wall of growing collaterals, hence they may also have a role in arteriogenesis (Heil et al., 2004) as they produce vascular endothelial cell growth factor (VEGF) (Couffinhal et al., 1999). Their specific role in arteriogenesis is still uncertain. Activated T lymphocytes secrete cytokines and they are able to modulate trafficking of other inflammatory cells, such as monocytes/macrophages.

A role for T lymphocytes in collateral vessel development was initially suggested by the observation of impaired collaterogenesis in athymic nude mice lacking T cells but with normal monocyte and macrophage counts (Couffinhal et al., 1999).

T lymphocytes are subdivided into two major subsets depending on the expression of the membrane antigens CD4 or CD8. Among these two main subsets, several subpopulations exist based on their functional properties hereafter referred as functional subsets. Naïve $CD4^+$ T cells can differentiate into T helper (Th) subsets based on their pattern of cytokine production. While T helper 1 lymphocytes produce interferon-γ (IFN-γ) but not interleukin-4 (IL-4), Th2 cells release IL-4 and IL-5 but not IFN-γ. T helper cells predominantly producing IL-17 (Th17) or IL-22 (Th22) have been described and are involved in chronic inflammation and in wound healing/skin diseases, respectively. In addition, other functional $CD4^+$ T cell subsets exist that display regulatory rather than effector functions. Three subsets of these regulatory T cells have been described: T regulatory 1 cells (Tr1) that function via the production of the cytokine IL-10, T helper 3 (Th3) cells producing TGF-β, and a population of $CD4^+CD25^+Foxp3^+$ T cells (Treg) that suppresses proliferation via a cell-contact-dependent mechanism.

Importantly, mice deficient in CD4 T lymphocytes showed reduced blood flow recovery after femoral artery ligation with concomitant decreased recruitment of macrophages during arteriogenesis (Stabile et al., 2003). Both $CD4^+$ and $CD8^+$ T cell subsets are necessary for efficient arteriogenesis. Infiltrating $CD4^+$ T cells contribute, at least in part, by actively releasing VEGF and chemokines thus recruiting circulating monocytes. In the absence of $CD4^+$ T lymphocytes, ischemia-induced inflammatory responses and collateral development are impaired compared to their wild-type (C57BL/6) littermates. Normal collateral response could be restored in CD4-deficient mice after

reconstitution with $CD4^+$ cells isolated from wild type animals. Moreover, $CD8^+$ T lymphocytes play a pivotal role in collateral response after femoral artery occlusion. Similarly to what observed in CD4-/- mice, animals lacking $CD8^+$ T cells displayed impaired development of collaterals, that could be restored by systemic administration of $CD8^+$ cells obtained from *wild-type* but not from IL-16 deficient mice (Stabile et al., 2006). Such finding, along with the capacity of IL-16 to act as a chemoattractant for $CD4^+$ cells, suggests a pivotal role for this cytokine in the cross-talk between inflammatory $CD8^+$ and $CD4^+$ T cells in the context of collateral vessels development.



Arteriogenesis. (A) Without significant stenosis, there is no significant pressure gradient over pre-existing collateral anastomoses, which are small and barely carry blood. (B) Development of a significant arterial obstruction leads to a drop in pressure and oxygen saturation distal in the vascular bed (purple-blue colour), while proximal pressure and oxygen saturation proximal remain normal (red colour). The pressure gradient over the collateral circulation increases fluid shear stress in these arterioles. (C) Close-up on the cellular level. The endothelium senses increased fluid shear stress via its cytoskeleton, transmembrane proteins (integrins, ion channels) and the glycocalyx. In an activated state, the endothelium expresses adhesion molecules (ICAM-1), to which circulating monocytes bind via their Mac-1 receptor. Monocytes transmigrate into perivascular tissue, differentiate to macrophages and secrete growth factors and cytokines that attract further monocytes and stimulate proliferation of smooth muscle cells and endothelial cells. (D) Adequately developed collateral arteries restore distal perfusion and provide sufficiently oxygenated blood to distal tissues. bFGF, basic fibroblast growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; ICAM, intercellular adhesion molecule; MCP, monocyte chemoattractant protein; MMPs, matrix metalloproteinases; TGFβ, transforming growth factor β.

Adapted from: **Schirmer S. H.et al., Heart. 2009**

AIM OF THE STUDY:

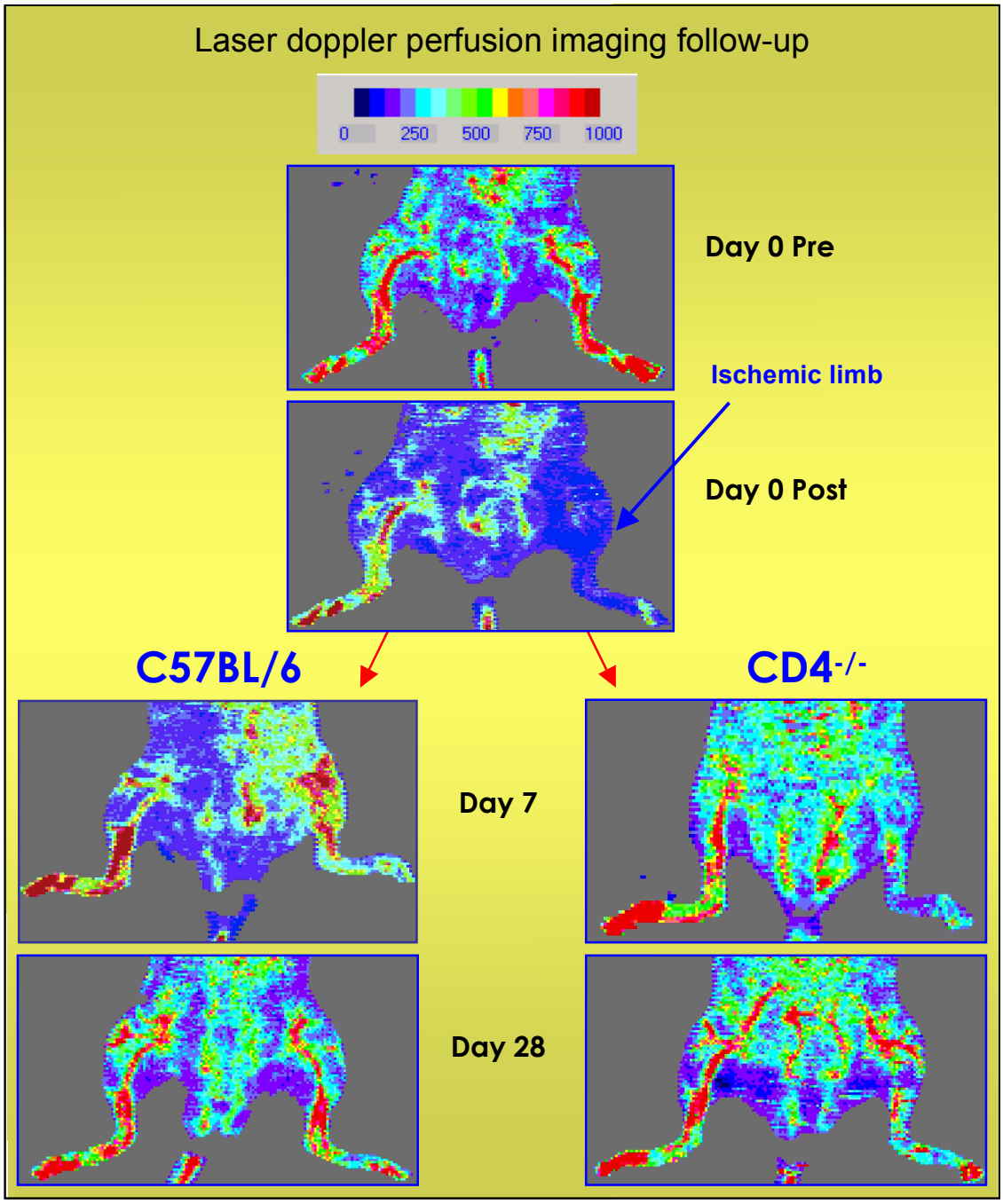
In this study we used a murine model of periferic ischemia (hindlimb ischemia) obtained through surgical ligation of left femoral artery to investigate the substantial role of different T lymphocyte subpopulations during the process of collateral vessels development.

While the pivotal role of macrophages that infiltrate sites of growing collaterals and secrete factors crucial for vascular remodelling is well established, the contribution of T lymphocytes has been unveiled only recently.

METHODS:

Angiogenic response to ischemia:

12 weeks old mice anaesthetized with intraperitoenal injection of xylazine (40 mg/kg) and ketamine (100 mg/kg), underwent surgical excision of the left femoral artery to induce unilateral hind limb ischemia. Mice body temperature was kept at 37°C during surgery and all post operative interventions. Blood flow recovery after surgical excision of the left femoral artery was periodically assessed over time using a LASER Doppler perfusion imaging (LDPI) (Moor Instruments); blood flow recovery between mid-calf and mid-foot region was measured. For consistent measurements, imaging was performed after limb hair removal and after heating to 37°C to minimize temperature variation. Calculated perfusion was expressed as a ratio of the ischemic to normal limb. Limb function and the rate and extent of the healing process were evaluated using a clinical derived score (Ambulatory Impairment Score) and a semi-quantitative score of tissue damage, respectively. Finally, we evaluated the number of arteries present within the upper thigh of 6 animals for each group 28 days after femoral artery ligation. Only arteries containing a continuous internal elastic lamina (as stained with Elastic Van Gieson) and a rim of muscular tissue were counted, this excluded all functional arterioles, which usually are considered resistance, rather than conductance vessels.



Histological analysis of the calf muscle:

A delayed and reduced blood flow recovery results in an increased incidence of autoamputation. These events were carefully recorded. At the end of follow up, in every animal, we performed histological analysis of the

calf muscle located downstream to the distal point of femoral artery ligation and tipically subjected to ischemia after surgery. Collateral development in the upper limb is functionally relevant only if it reduces the extent of ischemia in the distal limb and consequently prevents muscle damage. We estimated the extent of fibrosis and muscular atrophy by Sirius red tissue staining expecting to observe a significant increase in the amount of fibrosis and of muscle fibers atrophy, caused by the more prolonged time the muscle is subjected to severe ischemia in mice with deficient collateral vessel development.

Purification of CD4+ T cell subsets:

$CD4^+CD25^-$ effector T, $CD4^+CD25^+$ regulatory T and $CD4^+CD62L^+$ naïve T cells were isolated from age and gender-matched wild-type C57BL/6 spleens. Splenocytes were obtained by spleen homogenates (1 spleen yield typically 10^7 splenocytes) and used to obtain cellular subpopulations.

$CD4^+$ untouched cells were isolated by immunomagnetic selection (Miltenyi Biotechnology, Inc) followed by incubation with anti-CD25 beads, and negative (effector T cells) and positive (regulatory T cells) fractions were both collected.

For naïve T cells, immunomagnetic isolation was performed through negative selection using a $CD4^+CD62L^+$ T cell isolation kit (Miltenyi Biotechnology, Inc).

In order to analyze the role of Th1 and Th2 cells, naïve T cells were stimulated and cultured in polarizing conditions as follows:

The polarization torward Th1 cells was obtained incubating naïve T splenocytes in plates precoated with anti-CD3 (1,5 mg/ml) in the presence of anti-CD28 (1,5 mg/ml), anti-IL-4 (10 mg/ml) and IL-12 (10 ng/ml). For Th2 polarization naïve T cells were stimulated with the same doses of anti-CD3 and antiCD-28 in the presence of IL-4 (10 ng/ml) and anti-IFN-g (10 mg/ml).

To assess *in vitro* polarization, resting T cell lines were stimulated with phorbol-myristate-acetate (10 ng/ml) and ionomycin (1 mg/ml) for 4h. Cells were then fixed with 2% paraformaldehyde for 20' at 4°C and permeabilized by incubation with 0,5% saponine for 20' at room temperature before staining with fluorochrome-conjugated anti-IFN-g and anti-IL-4. The percentages of IFN-g-producing/IL4$^-$ Th1 and IL-4-producing/IFN-g$^-$ Th2 cells were measured by flow cytometric analysis fractions by means of positive selection following magnetic labeling (Miltenyi Biotechnology, Inc).

Rescue experiments by infusion of different subsets of T lymphocytes from C57BL/6 mice:

For reconstitution experiments 10^6 cells belonging to the appropriate purified CD4$^+$ T cell subset were injected i.v. at time of surgery.

RESULTS:

The role of the functional subsets of $CD4^+$ T cells Th1, Th2 and Treg cell has been investigated using CD4-deficient (CD4-/-) C57BL/6 mice, lacking the entire $CD4^+$ subsets repertoire. In order to assess the specific contribution of $CD4^+$ T cells functional subsets to collateral response, $CD4^{-/-}$ mice underwent surgical ligation of the left femoral artery and blood flow recovery kinetics were evaluated. Blood perfusion recovery was analyzed in wild-type and CD4-/- animals reconstituted or not with different $CD4^+$ T cell subsets freshly isolated from wild-type mice or generated *in vitro* as described in materials and methods.

Figure 1A shows the kinetic of blood flow in C57BL/6 and $CD4^{-/-}$ mice after left femoral artery surgical occlusion. In keeping with previous observations animals lacking $CD4^+$ cell subsets display impaired blood flow recovery as soon as day 3 post surgery. Blood flow recovery remains unpaired at later time points. The difference in blood flow between wild-type and CD4-/- animals is statistically significant at days 7, 14, 21 and 28 post surgery. As a result of reduced perfusion CD4-/- mice displayed altered limb function with subsequent ambulatory impairment (Figure 1B). Little acute ambulatory impairment was measurable in wild-type animals at days 3 and 7 post artery occlusion. Such impairment further decreased at later time points and virtually disappeared at day 28. In contrast ambulatory impairment in CD4-/- mice peaked at day 7 when it was 6 fold greater than that measured in wild-type animals.

Moreover CD4-/- mice only partially recovered ambulatory capacity at the end of the follow up time.

In the next series of experiments we compared the effect on blood flow recovery after artery occlusion of the administration of $CD4^+CD25^-$ effector T cells with the infusion of $CD4^+CD25^+$ regulatory T lymphocytes. $CD25^-$ and $CD25^+$ T cell subsets were purified from the spleen of wild-type mice and injected intravenously at time of surgery.

Effector but not regulatory T cells determined accelerated and augmented blood perfusion during the 4 week follow up (fig. 2A). These differences in blood perfusion recovery are consistent with the impairment in deambulation.

These results indicate that the capacity to foster collateral response relies exclusively on the effector T cell subset. However the inability of regulatory T cells to promote collateral vessels growth alone does not indicate whether this cells population acts as negative regulator of such process.

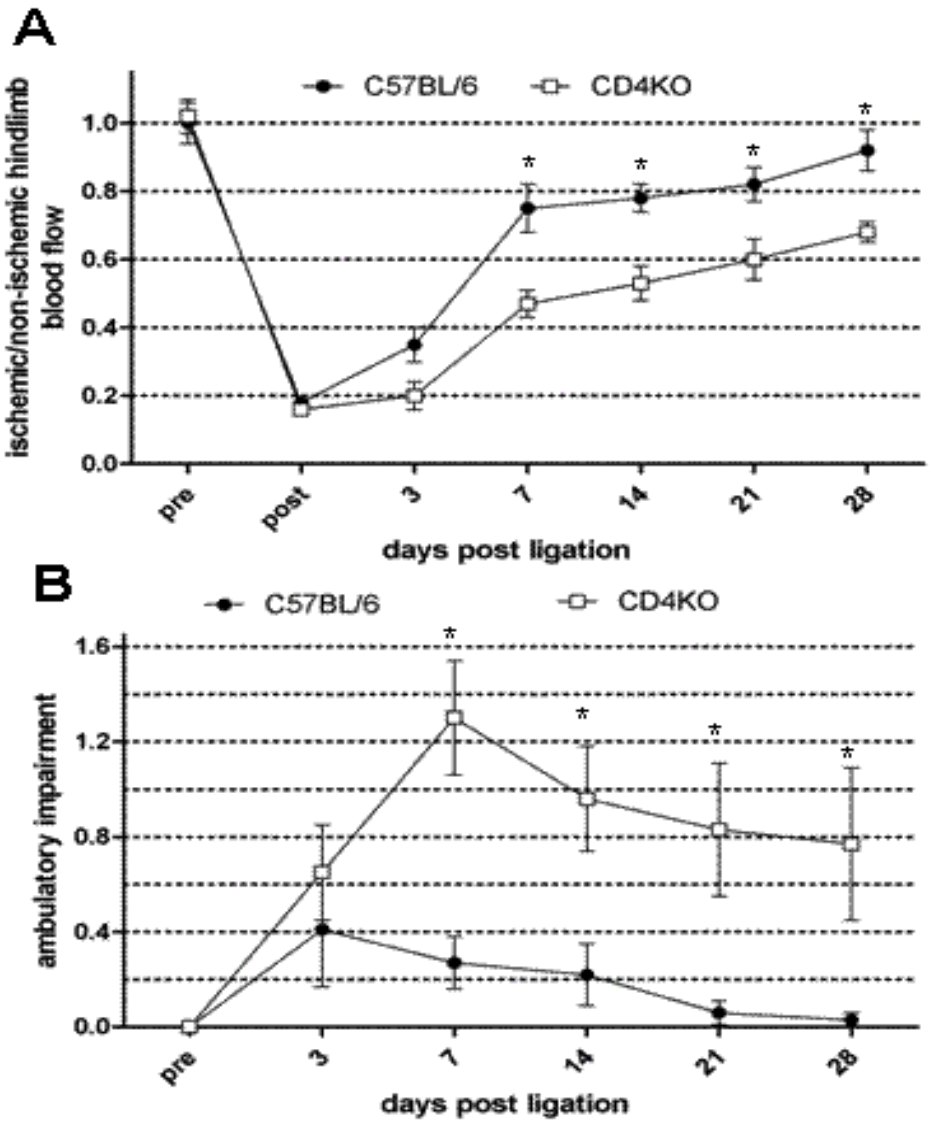


Fig. 1. Impaired collateral response to acute left hindlimb ischemia induced by surgical artery ligation in CD4-/- (CD4 KO) mice. A) ischemic limb blood flow recovery evaluated by laser Doppler perfusion imaging. Calculated perfusion is expressed as a ratio of the ischemic to normal limb that underwent the same surgical procedure except for femoral artery ligation. B) Semiquantitative assessment of impaired use of the ischemic limb expressed through the score below: 3=dragging of foot, 2=no dragging but no plantar flexion, 1=plantar flexion, and 0=flexing the toes to resist gentle traction on the tail.* = $p<0.05$ t Student's test.

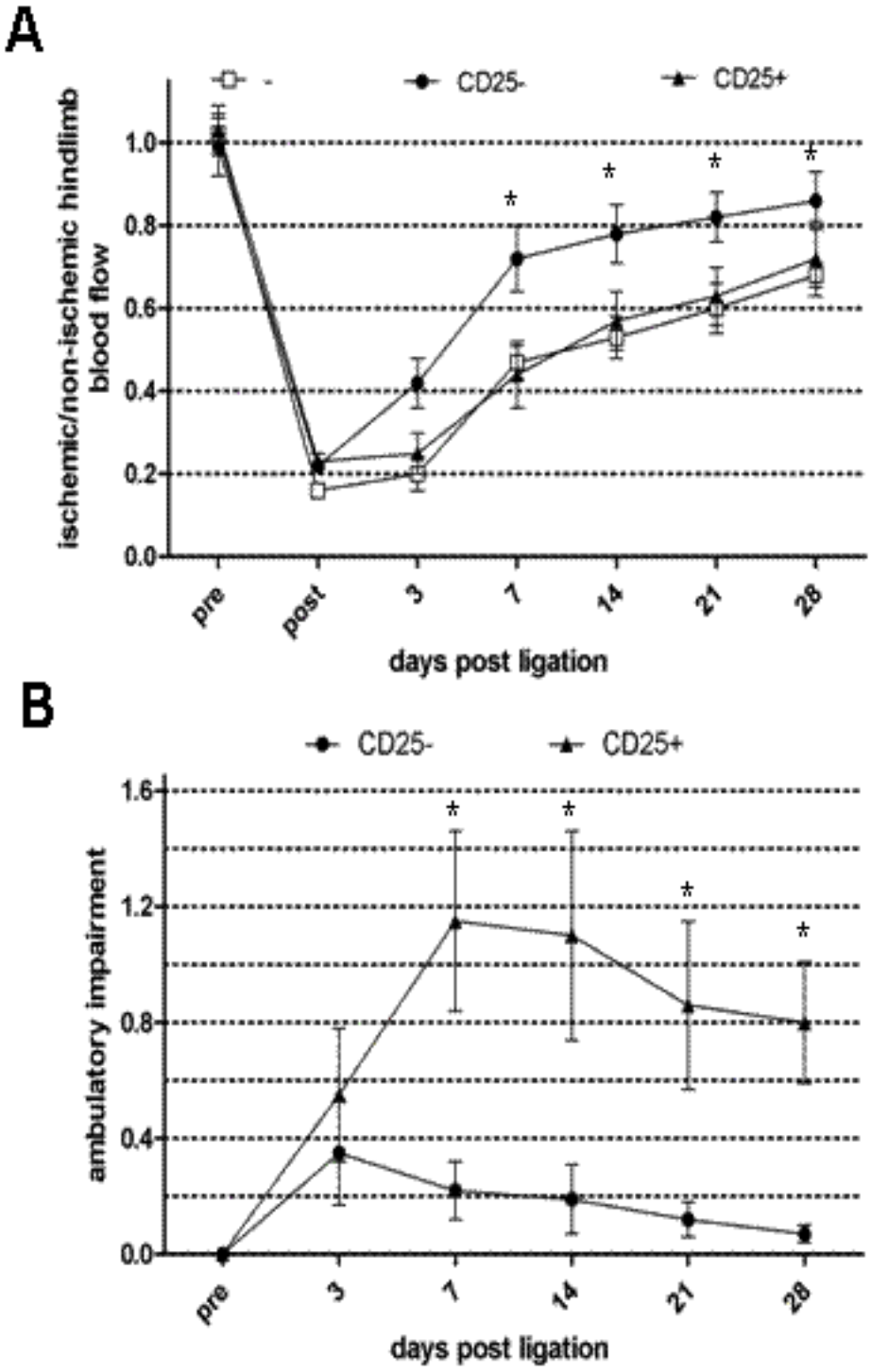


Fig. 2. Post-ischemical collateral vessels development recovery in CD4-/- (CD4KO) mice reconstituted with either effector T or regulatory T lymphocytes. A) Blood flow recovery in ischemic limb measured by laser Doppler perfusion imaging. Calculated perfusion is expressed as a ratio of the ischemic (left) to normal (right) limb that underwent the same surgical procedure except for femoral artery ligation. CD4-/- mice were reconstituted through intravenously perfusion of physiologic solution(-) or 10^6 effector T cells (CD25-) or regulatory T cells($CD25^+$) isolated from wild-type C57BL/6 mice spleen. B) Semiquantitative assessment of impaired use of the ischemic limb expressed through the score below: 3=dragging of foot, 2=no dragging but no plantar flexion, 1=plantar flexion, and 0=flexing the toes to resist gentle traction on the tail.* = $p<0.05$ t Student's test.

To gain a better insight into the role of regulatory T cells we initially compared the ability of the administration of naïve or memory CD4 T cells to modify collateral response to femoral artery occlusion in CD4-/- recipient mice. Of note, memory $CD4^+CD62L^-$ but not naïve $CD4^+CD62L^+$ T cells comprise the $CD4^+CD25^+$ Treg subset.

First of all we evaluated collateral vascular development and relative blood flow recovery and ischemic limb function in CD4-/- mice reconstituted with $CD4^+$ $CD62L^+$ naïve T cells. The infusion of naïve T cells, devoid of regulatory T cells, completely restored blood flow recovery in CD4 deficient mice (Figure 3A). In fact, kinetic of blood perfusion in naïve T cells reconstituted and wild-type mice were almost completely identical. In contrast the infusion of $CD4^+CD62L^-$ memory T cells that include the regulatory cells subset had no detectable effect on the recovery of blood flow. Next we asked whether the presence of Treg cells in the $CD4^+$ $CD62L^-$ memory population played a role in its capacity to regulate collateral vessels growth. To this aim $CD4^+CD62L^-$ memory T cells purified from wild type splenocytes were incubated with anti CD25 monoclonal antibody to deplete the $CD4^+CD25^+$ regulatory cell subset. The resulting cell population consisting in memory T cells devoid of regulatory T cells was then used to reconstitute CD4-/- mice who underwent femoral artery ligation. In these animals we observed complete recovery of the capacity to restore blood perfusion. These differences in blood perfusion recovery are consistent with the impairment in deambulation. These results suggest that Treg cells turn memory T cell population unable to foster collateral responses and point them out as possible negative regulators of

arteriogenesis.

In order to directly analyze the contribution of Th1 and Th2 cells in post-ischemic collateral response, purified $CD4^+CD62L^+$ naïve T cells from wild-type mice were cultured in polarizing conditions to generate IFN-γ-producing Th1 and IL-4-producing Th2 effector T cell lines. To ascertain the acquisition of functional characteristics of Th1 and Th2 cell subsets, cytokine production was assessed by intracellular staining with fluorochrome-conjugated anti IFN-γ and anti IL-4 antibodies.

T helper 1 and Th2 cell lines were then used to reconstitute CD4-/- mice at time of surgical femoral artery occlusion. Th1 but not Th2 are endowed with the capacity to support collaterals growth as assessed by the analysis of the kinetics of blood flow recovery and ambulatory impairment (figure 4A and 4B). Intrinsic impairment in blood flow recovery, characteristic of CD4-/- recipient mice, was not altered by the administration of Th2 cells, whereas Th1 markedly improved blood perfusion and limb function.

These results suggest that Th1 cells contribute as positive regulators of arteriogenesis while Th2 cells play a neutral or a negative role in such process.

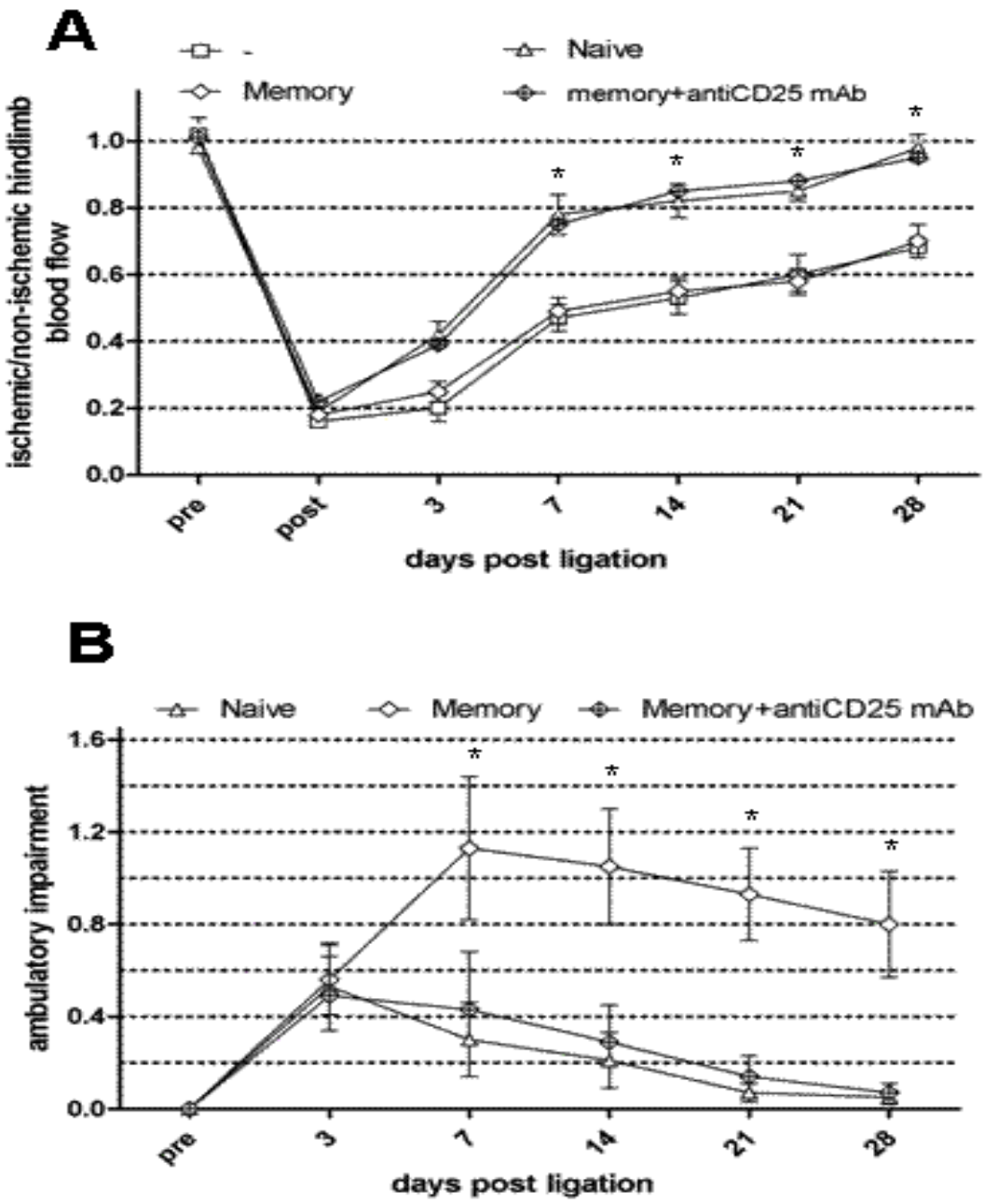


Fig. 3. CD4-/- mice reconstituted with either naïve T lymphocytes or CD62L$^-$ memory T cells depleted of CD25$^+$ Treg subset are able to re-establish the individual ability to mount an efficient collateral circulation following artery occlusion. A) Blood flow recovery in ischemic limb was measured by laser Doppler perfusion imaging. Calculated perfusion is expressed as a ratio of the ischemic (left) to normal (right) limb that underwent the same surgical procedure except for femoral artery ligation. CD4-/- mice were reconstituted by intravenous injection of physiologic solution(-) or 10^6 CD4$^+$ naïve or memory T lymphocytes or memory T cells after depletion of CD25$^+$ cells (memory + anti-CD25 mAb) isolated from wild-type C57BL/6 mice spleen. B) Semiquantitative assessment of limb function impairment. Individual mice were evaluated by the assignment of the following score: 3=dragging of foot, 2=no dragging but no plantar flexion, 1=plantar flexion, and 0=flexing the toes to resist gentle traction on the tail. *=$p<0.05$ t Student's test.

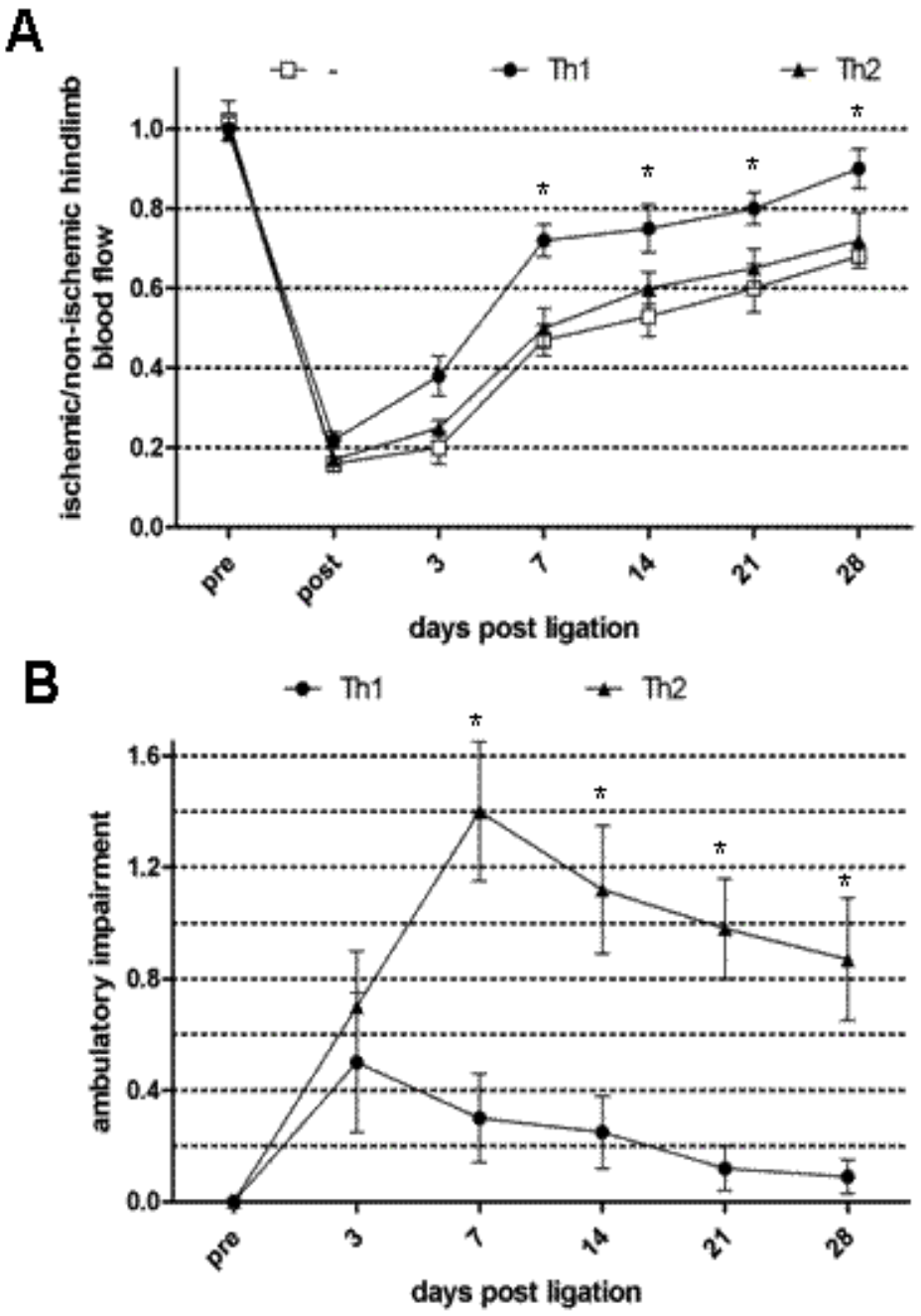


Fig. 4. Th1 but not Th2 cells are able to support collateral response giving rise to blood flow recovery in CD4-/- mice after surgical femoral artery ligation. A) Blood flow recovery in ischemic limb measured by laser Doppler perfusion imaging. Calculated perfusion is expressed as a ratio of the ischemic (left) to normal (right) limb that underwent the same surgical procedure except for femoral artery ligation. CD4-/- mice were reconstituted through intravenously perfusion of physiologic solution(-) or 10^6 T helper 1 (Th1) or T helper 2 (Th2) *in vitro* polarized from $CD4^+CD62L^+$ naïve T cells isolated by negative immunomagnetic selection from wild-type C57BL/6 mice spleen. B) Semiquantitative assessment of impaired use of the ischemic limb expressed through the score below: 3=dragging of foot, 2=no dragging but no plantar flexion, 1=plantar flexion, and 0=flexing the toes to resist gentle traction on the tail.* = $p<0.05$ t Student's test.

DISCUSSION AND CONCLUSIONS:

Compelling evidences point out a pivotal role for T lymphocytes in the orchestration of the inflammatory process underlying collateral vessels development. However, the specific contribution of different functional T cell subsets is to date still largely unknown.

A role for T lymphocytes in collateral vessel development was initially suggested by the observation of impaired collaterals development in athymic nude mice lacking T cells that bear the α/β T cell receptor but with normal monocyte and macrophage counts (Couffinhal et al., 1999).

Furthermore, Stabile and coworkers, using the hindlimb ischemia model in CD4- and CD8-deficient mice, described the essential role of $CD4^+$ and $CD8^+$ T cell subsets respectively for efficient arteriogenesis and development of collaterals (Stabile et al., 2003; Stabile et al., 2006).

Recently, Kumamoto and coworkers, using a murine model of viral infection, reported that $CD4^+$ T cells promote anti-viral $CD8^+$ T cell responses by increasing the input of naïve lymphocytes in the relevant draining lymph node. Although the molecular mechanism underlying such $CD4^+$ T cell function is still largely unknown, increased naïve T cell recruitment after viral infection is achieved in wild-type animals, at least in part, through the remodeling of the lymph node feeding arteriole to a larger diameter (Kumamoto et al., 2011).

In this study we aimed at characterizing the contribution of three important $CD4^+$ functional T cell subsets such as $CD25^+foxp3^+$ Treg lymphocytes, Th1 and

Th2 cells. Taking advantage of the murine hindlimb ischemia model for the study of arteriogenesis and collateral response to femoral artery occlusion, we initially compared the capacity to recover blood flow during a four weeks follow up time in wild-type and CD4-deficient knockout mice. In keeping with other observations, CD4 knockout mice displayed impaired capacity to mount efficient collateral response, resulting in reduced blood flow perfusion and impaired limb function. Thus, CD4-deficient mice were subsequently used as recipient to assess whether engraftment with specific $CD4^+$ T cell subsets, did rescue the capacity to recover blood flow following femoral artery occlusion.

A specific $CD4^+$ T cell subset expressing the transcription factor forkhead box (foxp3) and CD25 denominated $CD4^+CD25^+$ regulatory T cells (T regs) is specialized in the suppression of effector T cell function. Regulatory T cells contribute to the maintenance of immunologic self-tolerance and negative control of various immune responses (Sakaguchi, 2004).

To investigate the role of regulatory T cells, we initially performed rescue experiments in CD4-deficient recipient mice comparing the effects of the administration of two $CD4^+$ T cell subpopulations, defined by the expression of the low affinity IL-2 receptor CD25, on arteriogenesis. $CD4^+$ T lymphocytes expressing CD25 are highly enriched in regulatory T cells, whereas $CD25^-$ cells are considered effector lymphocytes. When CD4-deficient mice where reconstituted with effector cells, their capacity to recover blood perfusion after femoral artery ligation was restored. On the other hand, administration of $C4^+CD25^+$ T cells did not improve the kinetic of blood flow recovery. These

results indicated that $CD25^-$ effector but not $CD25^+$ regulatory T cells support inflammation leading to collateral vessels remodeling.

In order to obtain information about the interplay between these two cellular subsets, we then used a different approach. We took advantage of the fact that, by definition, regulatory T cells had encountered their specific antigen and therefore belong to the memory T cell population. As a consequence of the encounter with cognate antigen, naïve T cells downregulate surface expression of CD62L and are thus phenotypically identifiable as $CD62L^-$ cells by flow cytometry analysis. On the contrary, naïve T cell population retains surface CD62L expression and falls into the $CD62L^+$ cellular subset. Thus by simply separating $CD4^+$ T cells into naïve ($CD62L^+$) and memory ($CD62L^-$) subsets we obtained two populations: the first ($CD62L^+$, naïve) devoid of regulatory cells while the second ($CD62L^-$, memory) comprising both effector and regulatory cells. As shown in the rescue experiments illustrated in Fig. 3, naïve but not memory $CD4^+$ T cells were capable of restoring collateral responses thus indicating that the presence of Treg cells in the memory population might have exerted a dominant inhibitory effect on arteriogenesis. Consistent with such hypothesis, $CD62L^-$ memory cells depleted of the $CD25^+$ cell subset became effective in restoring collateral response.

The involvement of $CD4^+CD25^+$ Treg cells in the regulation of collateral response to ischemia has been recently proposed. Using CD28-deficient mice that lack T regs cell subset or by depleting Tregs in wild-type animals through the administration of an anti-CD25 antibody, Zouggari and coworkers recently showed increased efficiency in blood flow recovery after femoral artery

ligation (Zouggari et al., 2009). The authors speculated that the suppressive effect of T regs was due to their intrinsic inhibitory action on effector immune cell response. Interestingly, CD28-deficient mice that display severely reduced T regs numbers, are also characterized by enhanced type 1 effector T cell responses (Lenschow et al., 1996). Notably, several studies have indicated that Type 1 T cells are more susceptible than type 2 T lymphocytes to T regs-mediated suppression (Cosmi et al., 2004). It can be hypothesized that T regs might inhibit neovascularization by exerting preferential suppression of a particular functional subset of effector T lymphocytes, the IFN-γ-producing type 1 T cells.

Finally, we addressed the role of two major $CD4^+$ effector T cell subsets, namely Th1 and Th2. Although no formal demonstration is yet available, several observations indicate that type 1 T lymphocytes may foster post-ischemic neovascularization, whereas interleukin IL-4-producing type 2 cells may play a neutral or negative role. In support of this view it has been recently showed that 1) natural killer cells, that release large amounts of IFN-γ and are pivotal in the polarization of T helper cell responses toward the type 1 phenotype (Martin-Fontecha et al., 2004), play as essential positive regulators of post-ischemic neovascularization (van Weel et al., 2007) ; 2) IL-16 that is crucial for normal recruitment of inflammatory cells at site of collateral vessels development (Stabile et al., 2006), has been also shown to preferentially recruit Th1 lymphocytes (Lynch et al., 2003); 3) reduced collateral response is evident in Balb/c compared to C57BL/6 mouse strain (Helisch et al., 2006). Interestingly, Balb/c mice have higher numbers of circulating Tregs compared

to C57BL/6 (Chen et al., 2005) and are prone to mount type 2 T cell responses while C57BL/6 mice display a Th1-skewed phenotype.

Our experimental approach consisted in generating T cell lines *in vitro* from freshly isolated naïve CD4+ T cells by polyclonal stimulation in the presence of cytokines promoting the polarization of T cells toward Th1 or Th2 phenotypes. Before use, the functional phenotype of these cell lines defined as T helper 1 when producing large amounts of INF-γ but low or none IL-4 or IL-17, or as T helper 2 when releasing large amounts of IL-4 but low or none IFN-γ was assessed *in vitro*.

Rescue experiments involving the transplantation of *in vitro* polarized Th1 cells showed that reconstitution with this specific subset alone was effective in restoring almost completely the capacity of mice to recover blood flow and limb function, after the surgical procedure of femoral artery ligation. On the other hand, the administration of Th2 polarized cells did not alter the CD4-/- phenotype with respect to the capacity to mount collateral response to artery occlusion. These results suggest that T helper 1 may play as positive regulators of collateral responses. In contrast Th2 cells may have a neutral role in arteriogenesis although we cannot formally exclude that an hypothetical inhibitory effect of Th2 might have not be revealed because of the intrinsic impairment of the CD4-/- recipient mice in developing collateral circulation.

The identification of specific circulating cellular subsets playing either as positive or negative regulators of collaterals growth may be exploited for the identification of novel easily detectable molecular markers for the assessment of the individual capacity to compensate vascular occlusion, which could

potentially represent reliable markers of individual cardiovascular risk. Hopefully, they could also become new molecular targets for the design of novel therapeutic approaches for peripheral and cardiac ischemic diseases.

REFERENCES:

Arras M, Ito WD, Scholz D, Winkler B, Schaper J, Schaper W. (1998). Monocyte Activation in Angiogenesis and Collateral Growth in the Rabbit Hindlimb. *J Clin Invest.* 101:40-50.

Baffour R, Berman J, Garb JL, Rhee SW, Kaufman J, Friedmann P. (1992). Enhanced angiogenesis and growth of collaterals by in vivo administration of recombinant basic fibroblast growth factor in a rabbit model of acute lower limb ischemia: dose-response effect of basic fibroblast growth factor. *J Vasc Surg.* 16:181-191.

Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marme D. (1996). Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood*. 87:3336-3343.

Bergmann CE, Hoefer IE, Meder B, Roth H, van Royen N, Breit SM, Jost MM, Aharinejad S, Hartmann S, Buschmann IR. (2006). Arteriogenesis depends on circulating monocytes and macrophage accumulation and is severely depressed in op/op mice. *J Leukoc Biol*. 80:59-65.

Bernardini G, Ribatti D, Spinetti G, Morbidelli L, Ziche M, Santoni A, Capogrossi MC, Napolitano M. (2003). Analysis of the role of chemokines in angiogenesis. *J Immunol Methods*. 273:83-101.

Boutin AT, Weidemann A, Fu Z, Mesropian L, Gradin K, Jamora C, Weisener M, Eckardt KU, Koch CJ, Ellies LG, Haddad G, Haase VH, Simon MC, Poellinger L, Powell FL, Johnson RS. (2008). Epidermal sensing of oxygen is essential for systemic hypoxic response. *Cell*. 133:223-234.

Buschmann I, Schaper W. (1999). Arteriogenesis Versus Angiogenesis: Two Mechanisms of Vessel Growth. *News Physiol Sci*. 14:121-125.

Cai W, Vosschulte R, Afsah-Hedjri A, Koltai S, Kocsis E, Scholz D, Kostin S, Schaper W, Schaper J. (2000). Altered balance between extracellular proteolysis and antiproteolysis is associated with adaptive coronary arteriogenesis. *J Mol Cell Cardiol.* 32:997-1011.

Cai WJ, Kocsis E, Luo X, Schaper W, Schaper J. (2004). Expression of endothelial nitric oxide synthase in the vascular wall during arteriogenesis. *Org Lett*. 6:4203-4205.

Cao R, Brakenhielm E, Pawliuk R, Wariaro D, Post MJ, Wahlberg E, Leboulch P, Cao Y. (2003). Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. *Nat Med*. 9:604-613.

Cao Y, Li CY, Moeller BJ, Yu D, Zhao Y, Dreher MR, Shan S, Dewhirst MW.(2005). Observation of incipient tumor angiogenesis that is independent of hypoxia and hypoxia inducible factor-1 activation. *Cancer Res*. 65:5498-5505.

Cao Y.(2009). Monotherapy versus combination therapy of angiogenic and arteriogenic factors for the treatment of ischemic disorders. *Curr Mol Med*. 9:967-972.

Carmeliet P. (2000). VEGF gene therapy: stimulating angiogenesis or angioma-genesis?. *Nat Med*. 6:1102-1103.

Carmeliet P. (2003). Angiogenesis in health and disease. *Nat Med*. 9:653-660.

Chachisvilis M, Zhang YL, Frangos JA. (2006). G protein-coupled receptors sense fluid shear stress in endothelial cells. *Proc Natl Acad Sci U S A*. 103:15463-15468.

Chen KD, Li YS, Kim M, Li S, Yuan S, Chien S, Shyy JY. (1998). Mechanotransduction in response to shear stress. Roles of receptor tyrosine kinases, integrins, and Shc. *J Biol Chem*. 274:18393-18400.

Chen X, Oppenheim JJ, Howard OMZ. (2005). BALB/c mice have more CD4+CD25+ T regulatory cells and show greater susceptibility to suppression

of their CD4+CD25- responder T cells than C57BL/6 mice. *J Leukoc Biol*. 78:114-121.

Chung AS, Lee J, Ferrara N. (2010). Targeting the tumour vasculature: insights from physiological angiogenesis. *Nat Rev Cancer*. 10:505-514.

Cosmi L, Liotta F, Angeli R, Mazzinghi B, Santarlasci V, Manetti R, Lasagni L, Vanini V, Romagnani P, Maggi E, Annunziato F, Romagnani S. (2004). Th2 cells are less susceptible than Th1 cells to the suppressive activity of CD25+ regulatory thymocytes because of their responsiveness to different cytokines. Blood. 103:3117-3121

Couffinhal T, Silver M, Kearney M, Sullivan A, Witzenbichler B, Magner M, Annex B, Peters K, Isner JM. (1999). Impaired Collateral Vessel Development Associated With Reduced Expression of Vascular Endothelial Growth Factor in ApoE-/- Mice. *Circulation*. 99:3188-3198.

Cox R. (1979). Physiology and hemodynamics of the macrocirculation. In: Stehbens W, eds. Hemodynamics and the Blood Vessel Wall. *Springfield, Ill: Charles C. Thomas;* 75–156.

Davies PF, Barbee KA, Volin MV, Robotewskyj A, Chen J, Joseph L, Griem ML, Wernick MN, Jacobs E, Polacek DC, dePaola N, Barakat AI. (1997). Spatial

relationships in early signaling events of flow-mediated endothelial mechanotransduction. *Annu Rev Physiol*. 59:527-549.

Deindl E, Hoefer IE, Fernandez B, Barancik M, Heil M, Strniskova M, Schaper W. (2003). Involvement of the Fibroblast Growth Factor System in Adaptive and Chemokine-Induced Arteriogenesis. *Circ Res*. 92:561-568.

Eliceiri BP, Paul R, Schwartzberg PL, Hood JD, Leng J, Cheresh DA. (1999). Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. *Mol Cell*. 4:915-924.

Emanueli C, Salis MB, Pinna A, Graiani G, Manni L, Madeddu P. (2002). Nerve growth factor promotes angiogenesis and arteriogenesis in ischemic hindlimbs. *Circulation*. 106:2257–2262.

Eriksson A, Cao R, Roy J, Tritsaris K, Wahlestedt C, Dissing S, Thyberg J, Cao Y. (2003). Small GTP-binding protein Rac is an essential mediator of vascular endothelial growth factor-induced endothelial fenestrations and vascular permeability. *Circulation*. 107:1532-1538.

Ferguson JE 3rd, Kelley RW, Patterson C. (2005). Mechanisms of endothelial differentiation in embryonic vasculogenesis. *Arterioscler Thromb Vasc Biol*. 25:2246-2254.

Fernandez B, Buehler A, Wolfram S, Kostin S, Espanion G, Franz WM, Niemann H, Doevendans PA, Schaper W, Zimmermann R. (2000). Transgenic myocardial overexpression of fibroblast growth factor-1 increases coro-nary artery density and branching. *Circ Res*. 87:207–213.

Fischer C, Schneider M, Carmeliet P. (2006). Principles and therapeutic implications of angiogenesis, vasculogenesis and arteriogenesis. *Handb Exp Pharmacol*. 176 Pt 2:157-212.

Hänze J, Weissmann N, Grimminger F, Seeger W, Rose F. (2007). Cellular and molecular mechanisms of hypoxia-inducible factor driven vascular remodeling. *Thromb Haemost*. 97:774-787.

Heil M, Clauss M, Suzuki K, Buschmann IR, Willuweit A, Fischer S, Schaper W. (2000). Vascular endothelial growth factor (VEGF) stimulates monocyte migration through endothelial monolayers via increased integrin expression. *Eur J Cell Biol*. 79:850-857.

Heil M, Eitenmüller I, Schmitz-Rixen T, Schaper W. (2006). Arteriogenesis versus angiogenesis: similarities and differences. *J Cell Mol Med*. 10:45-55.

Heil M, Schaper W. (2004). Influence of Mechanical, Cellular, and Molecular Factors on Collateral Artery Growth (Arteriogenesis). *Circ Res*. 95:449-458.

Heil M, Schaper W. (2004). Pathophysiology of collateral development. *Coron Artery Dis*. 15:373-378.

Heil M, Ziegelhoeffer T, Wagner S, Fernandez B, Helisch A, Martin S, Tribulova S, Kuziel WA, Bachmann G, Schaper W. (2004). Collateral Artery Growth (Arteriogenesis) After Experimental Arterial Occlusion Is Impaired in Mice Lacking CC-Chemokine Receptor-2. *Circ Res*. 94:671-677.

Helisch A, Wagner S, Khan N, Drinane M, Wolfram S, Heil M, Ziegelhoeffer T, Brandt U, Pearlman JD, Swartz HM, Schaper W. (2006). Impact of Mouse Strain Differences in Innate Hindlimb Collateral Vasculature. *Arterioscler Thromb Vasc Biol*. 26:520-526.

Hellström M, Kalén M, Lindahl P, Abramsson A, Betsholtz C. (1999). Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development*. 126: 3047-55.

Hoefer IE, Piek JJ, Pasterkamp G. (2006). Pharmaceutical interventions to influence arteriogenesis: new concepts to treat ischemic heart disease. *Curr Med Chem*. 13:979-987.

Hoefer IE, van Royen N, Buschmann IR, Piek JJ, Schaper W. (2001). Time course of arteriogenesis following femoral artery occlusion in the rabbit. Cardiovascular *Research*. 49:609-617.

Hoefer IE, van Royen N, Jost MM. (2006). Experimental models of arteriogenesis: differences and implications. *Lab Anim (NY)*. 35:36-44.

Hoefer IE, van Royen N, Rectenwald JE, Bray EJ, Abouhamze Z, Moldawer LL, Voskuil M, Piek JJ, Buschmann IR, Ozaki CK. (2002). Direct evidence for tumor necrosis factor signaling in arteriogenesis. *Circulation*.105:1639 –1641.

Hoefer IE, van Royen N, Rectenwald JE, Deindl E, Hua J, Jost M, Grundmann S, Voskuil M, Ozaki CK, Piek JJ, Buschmann IR. (2004). Arteriogenesis proceeds via ICAM-1/Mac-1- mediated mechanisms. *Circ Res*. 94:1179-1185.

Iruela-Arispe ML, Davis GE. (2009). Cellular and molecular mechanisms of vascular lumen formation. *Dev Cell*. 16:222-231.

Ito WD, Arras M, Winkler B, Scholz D, Schaper J, Schaper W. (1997). Monocyte Chemotactic Protein-1 Increases Collateral and Peripheral Conductance After Femoral Artery Occlusion. *Circ Res*. 80:829-837.

Jalali S, del Pozo MA, Chen K, Miao H, Li Y, Schwartz MA, Shyy JY, Chien S. (2001). Integrin-mediated mechanotransduction requires its dynamic

interaction with specific extracellular matrix (ECM) ligands. *Proc Natl Acad Sci U S A*. 98:1042-1046.

Jin ZG, Ueba H, Tanimoto T, Lungu AO, Frame MD, Berk BC. (2003). Ligand-independent activation of vascular endothelial growth factor receptor 2 by fluid shear stress regulates activation of endothelial nitric oxide synthase. *Circ Res*. 93:354-363.

Jones EA, le Noble F, Eichmann A. (2006). What determines blood vessel structure? *Genetic prespecification vs. hemodynamics.Physiology (Bethesda)*. 21:388-395.

Kosaki K, Ando J, Korenaga R, Kurokawa T, Kamiya A. (1998). Fluid shear stress increases the production of granulocyte-macrophage colony-stimulating factor by endothelial cells via mRNA stabilization. *Circ Res*. 82:794-802.

Kumamoto Y, Mattei LM, Sellers S, Payne GW, Iwasaki A. (2011). CD4+ T cells support cytotoxic T lymphocyte priming by controlling lymph node input. *Proc Natl Acad Sci U S A*. 108:8749-8754.

Lee CW, Stabile E, Kinnaird T, Shou M, Devaney JM, Epstein SE, Burnett MS. (2004). Temporal patterns of gene expression after acute hindlimb ischemia in mice: insights into the genomic program for collateral vessel development. *J Am Coll Cardiol*. 43:474-482.

Lendahl U, Lee KL, Yang H, Poellinger L. (2009). Generating specificity and diversity in the transcriptional response to hypoxia. *Nat Rev Genet*.10:821-832.

Lenschow DJ, Herold KC, Rhee L, Patel B, Koons A, Qin HY, Fuchs E, Singh B, Thompson CB, Bluestone JA. (1996). CD28/B7 Regulation of Th1 and Th2 Subsets in the Development of Autoimmune Diabetes. *Immunity*. 5:285-293.

Lifton RP, Gharavi AG, Geller DS. (2001). Molecular mechanisms of human hypertension. *Cell*. 104:545-556.

Liu ZJ, Shirakawa T, Li Y, Soma A, Oka M, Dotto GP, Fairman RM, Velazquez OC, Herlyn M. (2003). Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis. *Mol Cell Biol*. 23: 14 –25.

Luo D, Luo Y, He Y, Zhang H, Zhang R, Li X, Dobrucki WL, Sinusas AJ, Sessa WC, Min W. (2006). Differential functions of tumor necrosis factor receptor 1 and 2 signaling in ischemia-mediated arteriogenesis and angiogenesis. *Am J Pathol*. 169:1886-1898.

Luttun A, Carmeliet P. (2003). De novo vasculogenesis in the heart. *Cardiovasc Res.* 58:378-389.

Lynch EA, Heijens CAW, Horst NF, Center DM, Cruikshank WW. (2003). Cutting Edge: IL-16/CD4 Preferentially Induces Th1 Cell Migration: Requirement of CCR5. *J Immunol*. 171:4965-4968.

Makino Y, Cao R, Svensson K, Bertilsson G, Asman M, Tanaka H, Cao Y, Berkenstam A, Poellinger L. (2001). Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. *Nature*. 414:550-554.

Martin-Fontecha A, Thomsen LL, Brett S, Gerard C, Lipp M, Lanzavecchia A, Sallusto F. (2004). Induced recruitment of NK cells to lymph nodes provides IFN-[gamma] for TH1 priming. *Nat Immunol*. 5:1260-1265.

Nilius B, Droogmans G. (2001). Ion channels and their functional role in vascular endothelium. *Physiol Rev*. 81:1415-1459.

Pardanaud L, Yassine F, Dieterlen-Lievre F. (1989). Relationship between vasculogenesis, angiogenesis and haemopoiesis during avian ontogeny. *Development*. 103:473-485.

Péault B. (2010). Hemangioblasts: back to the future?. *Blood*. 116:2864-2865.

Pelosi E, Valtieri M, Coppola S, Botta R, Gabbianelli M, Lulli V, Marziali G, Masella B, Müller R, Sgadari C, Testa U, Bonanno G, Peschle C. (2002). Identification of the hemangioblast in postnatal life. *Blood*. 100:3203-3208.

Pipp F, Boehm S, Cai WJ, Adili F, Ziegler B, Karanovic G, Ritter R, Balzer J, Scheler C, Schaper W, Schmitz-Rixen T. (2004). Elevated fluid shear stress enhances postocclusive collateral artery growth and gene expression in the pig hind limb. *Arterioscler Thromb Vasc Biol*. 24:1664-1668.

Pipp F, Heil M, Issbrucker K, Ziegelhoeffer T, Martin S, van den HJ, Weich H, Fernandez B, Golomb G, Carmeliet P, Schaper W, Clauss M. (2003). VEGFR-1-selective VEGF homologue PlGF is arteriogenic: evidence for a monocyte-mediated mechanism. *Circ Res*. 92:378-385.

Polverini PJ. (1997). Role of the macrophage in angiogenesis-dependent diseases. *EXS*. 79:11-28.

Risau W, Flamme I. (1995). Vasculogenesis. *Annu Rev Cell Dev Biol*. 11:73-91.

Risau W. (1997). Mechanisms of angiogenesis. *Nature*. 386:671-674.

Rissanen TT, Markkanen JE, Arve K, Rutanen J, Kettunen MI, Vajantol, Jauhiainen S, Cashion L, Gruchala M, Narvanen O, Taipale P, Kauppinen RA, Rubanyi GM, Yla-Herttuala S. (2003). Fibroblast growth factor 4 induces vascular permeability, angiogenesis and arteriogenesis in a rabbit hindlimb ischemia model. *Faseb J*.17:100–102.

Sakaguchi S. (2004). Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol. 22:531-562

Saunders WB, Bohnsack BL, Faske JB, Anthis NJ, Bayless KJ, Hirschi KK, Davis GE. (2006). Coregulation of vascular tube stabilization by endothelial cell TIMP-2 and pericyte TIMP-3. *J Cell Biol*. 175:179-191.

Schaper J, König R, Franz D, Schaper W. (1976). The endothelial surface of growing coronary collateral arteries. Intimal margination and diapedesis of monocytes. A combined SEM and TEM study. *Virchows Arch A Pathol Anat Histol.* 370:193-205.

Schaper W, Buschmann I. (1999). Arteriogenesis, the good and bad of it. *Cardiovasc Res*. 43:835-837.

Schaper W, Scholz D. (2003). Factors regulating arteriogenesis. *Arterioscler Thromb Vasc Biol*. 23:1143-1151.

Scheel KW, Fitzgerald EM, Martin RO, Larsen RA. (1979). The possible role of mechanical stresses on coronary collateral development during gradual coronary occlusion. In: Schaper W, eds. The Pathophysiology of Myocardial Perfusion. *Amsterdam: Elsevier/North-Holland*; 489–518.

Scholz D, Ito W, Fleming I, Deindl E, Sauer A, Wiesnet M, Busse R, Schaper J, Schaper W. (2000). Ultrastructure and molecular histology of rabbit hind-limb collateral artery growth (arteriogenesis). *Virchows Arch*. 436:257-270.

Scholz D, Ziegelhoeffer T, Helisch A, Wagner S, Friedrich C, Podzuweit T, Schaper W. (2002). Contribution of arteriogenesis and angiogenesis to post-tocclusive hindlimb perfusion in mice. *J Mol Cell Cardiol*. 34: 775–787.

Semenza GL. (2007). Life with oxygen. *Science*. 318:62-64.

Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science*. 219:983-985.

Stabile E, Burnett MS, Watkins C, Kinnaird T, Bachis A, la Sala A, Miller JM, Shou M, Epstein SE, Fuchs S. (2003). Impaired arteriogenic response to acute hindlimb ischemia in CD4-knockout mice. *Circulation*. 108:205-210.

Stabile E, Kinnaird T, la Sala A, Hanson SK, Watkins C, Campia U, Shou M, Zbinden S, Fuchs S, Kornfeld H, Epstein SE, Burnett MS. (2006). CD8+ T Lymphocytes Regulate the Arteriogenic Response to Ischemia by Infiltrating the Site of Collateral Vessel Development and Recruiting CD4+ Mononuclear Cells Through the Expression of Interleukin-16. *Circulation*. 113:118-124.

Tirziu D, Moodie KL, Zhuang ZW, Singer K, Helisch A, Dunn JF, Li W, Singh J, Simons M. (2005). Delayed arteriogenesis in hypercholesterolemic mice. *Circulation*. 112:2501-2509.

Tirziu D, Simons M. (2005). Angiogenesis in the human heart: gene and cell therapy. *Angiogenesis*. 8:241-251.

Unger EF, Banai S, Shou M, Lazarous DF, Jaklitsch MT, Sheinowitz M, Correa R, Klingbeil C, Epstein SE. (1994). Basic fibroblast growth factor enhances myocardial collateral flow in a canine model. *Am J Physiol*. 266:H1588 –H1595.

van Royen N, Hoefer I, Bottinger M, Hua J, Grundmann S, Voskuil M, Bode C, Schaper W, Buschmann I, Piek JJ. (2003). Local Monocyte Chemoattractant Protein-1 Therapy Increases Collateral Artery Formation in Apolipoprotein E-Deficient Mice but Induces Systemic Monocytic CD11b Expression, Neointimal Formation, and Plaque Progression. *Circ Res*. 92:218-225.

van Royen N, Hoefer I, Buschmann I, Heil M, Kostin S, Deindl E, Vogel S, Korff T, Augustin H, Bode C, Piek JJ, Schaper W. (2002). Exogenous application of transforming growth factor beta 1 stimulates arteriogenesis in the peripheral circulation. *FASEB J*. 16:432-434.

van Weel V, Toes REM, Seghers L, Deckers MML, de Vries MR, Eilers PH, Sipkens J, Schepers A, Eefting D, van Hinsbergh VWM, van Bockel JH, Quax PHA.

(2007). Natural Killer Cells and CD4+ T-Cells Modulate Collateral Artery Development. *Arterioscler Thromb Vasc Biol*. 27:2310-2318.

Voskuil M, van Royen N, Hoefer IE, Seidler R, Guth BD, Bode C, Schaper W, Piek JJ, Buschmann IR. (2003). Modulation of collateral artery growth in a porcine hindlimb ligation model using MCP-1. *Am J Physiol Heart Circ Physiol*. 284:H1422-H1428.

Wolf C, Cai WJ, Vosschulte R, Koltai S, Mousavipour D, Scholz D, Afsah-Hedjri A, Schaper W, Schaper J. (1998). Vascular remodeling and altered protein expression during growth of coronary collateral arteries. *J Mol Cell Cardiol*. 30:2291-2305.

Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. (2000). Vascular-specific growth factors and blood vessel formation. *Nature*. 407:242-248.

Zeeb M, Strilic B, Lammert E. (2010). Resolving cell-cell junctions: lumen formation in blood vessels. *Curr Opin Cell Biol*. 22:626-632.

Zouggari Y, Ait-Oufella H, Waeckel L, Vilar J, Loinard C, Cochain C, Récalde A, Duriez M, Levy BI, Lutgens E, Mallat Z, Silvestre JS. (2009). Regulatory T cells modulate postischemic neovascularization. *Circulation*. 120:1415-1425.